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Molecular evidence for the monophyly of East Asian groups of Cyprinidae (Teleostei: Cypriniformes) derived from the nuclear recombination activating gene 2 sequences

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Abstract

The family Cyprinidae is one of the largest families of fishes in the world and a well-known component of the East Asian freshwater fish fauna. However, the phylogenetic relationships among cyprinids are still poorly understood despite much effort paid on the cyprinid molecular phylogenetics. Original nucleotide sequence data of the nuclear recombination activating gene 2 were collected from 109 cyprinid species and four non-cyprinid cypriniform outgroup taxa and used to infer the cyprinid phylogenetic relationships and to estimate node divergence times. Phylogenetic reconstructions using maximum parsimony, maximum likelihood, and Bayesian analysis retrieved the same clades, only branching order within these clades varied slightly between trees. Although the morphological diversity is remarkable, the endemic cyprinid taxa in East Asia emerged as a monophyletic clade referred to as Xenocypridini. The monophyly for the subfamilies including Cyprininae and Leuciscinae, as well as the tribes including Labeonini, Gobionini, Acheilognathini, and Leuciscini, was also well resolved with high nodal support. Analysis of the RAG2 gene supported the following cyprinid molecular phylogeny: the Danioninae is the most basal subfamily within the family Cyprinidae and the Cyprininae is the sister group of the Leuciscinae. The divergence times were estimated for the nodes corresponding to the principal clades within the Cyprinidae. The family Cyprinidae appears to have originated in the mid-Eocene in Asia, with the cladogenic event of the key basal group Danioninae occurring in the early Oligocene (about 31–30 MYA), and the origins of the two subfamilies, Cyprininae and Leuciscinae, occurring in the mid-Oligocene (around 26 MYA).

Keywords: Cyprinidae; The recombination activating gene 2; Nuclear phylogeny; East Asia

1. Introduction

Cyprinidae, one of the most diverse freshwater fish groups in the world, achieve their maximal diversity in Asiatic waters (Fu et al., 2003). In Asia, there exists about 1200 species, with the greatest generic and species diversity in East and Southeast Asia (Bănărescu and Coad, 1991; Rainboth, 1991). The cyprinid fauna in East Asia includes many endemic subfamilies or informal subgroupings, e.g. cultrins, xenocyprins, and squaliobarbins etc. (Chen, 1998; Howes, 1991). This tremendous species diversity of East Asian cyprinids makes this group especially important for many comparative evolutionary investigations relating to the timing and patterns of diversification in freshwater fishes of Asia. Despite the wealth of scientific data represented by the East Asian cyprinids, few clear advances have been made in understanding their evolutionary relationships. Furthermore, the lack of phylogenetic studies on Asian cyprinids has resulted in a limited understanding of higher-level relationships and classification within the family Cyprinidae.

The number and interrelationships of the subfamilies within the family Cyprinidae remained controversial in spite of considerable systematics studies based on morphological or anatomical characters (Berg, 1940; Chen et al., 1984; Chu, 1935; Gosline, 1978; Howes, 1991; Wu, 1964). In previous morphological investigations, the East Asian

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cyprinids were grouped into many different subfamilies, and it is clear that the number of subfamilies recognized was greatly influenced by the diversity and placement of the included East Asian cyprinids. According to the latest taxonomic revision proposed by Chen (1998), the family Cyprinidae was divided into 12 subfamilies: Danioninae, Leuciscinae, Cultrinae, Xenocyprinae, Hypophthalmichthyinae, Cobioninae, Gobiobotinae, Acheilognathinae, Barbinae, Labeoninae, Schizothoracinae, and Cyprininae. However, some of these subfamilies were supported by only a few characters.

Recent investigations of Cyprinidae using molecular data and phylogenetic methods have focused on relationships among European (Briolay et al., 1998; Durand et al., 2002; Gilles et al., 1998, 2001; Machordom and Doadrio, 2001; Zardoya and Doadrio, 1999), North American (Dowling et al., 2002) and Asian cyprinids (Cunha et al., 2002; He et al., 2004; Liu and Chen, 2003). These molecular analyses have revealed certain systematic inconsistencies in the relationships and classification of the European cyprinids (Kotlík et al., 2004), as well as in those studies of the East Asian species (Cunha et al., 2002; He et al., 2004; Liu and Chen, 2003), indicating that large-scale molecular evidence does not support the monophyly of certain traditional taxonomic subfamilies within cyprinids as proposed by previous authors (Chen, 1998; Howes, 1991). Although molecular studies have shed light on taxonomic problems within the family, the interrelationships of the Tincinae, Danioninae, and Acheilognathinae remain unclear because their positions change from one gene to another or their relationships are consistently revealed with very low bootstrap support (Gilles et al., 1998; Liu and Chen, 2003; Zardoya and Doadrio, 1999). To date, molecular phylogenies of East Asian cyprinids have had limited taxon coverage and have relied extensively on mtDNA sequences. Given that taxon sampling has been clearly identified as an important source of error in analyses by revealing inaccurate phylogenetic relationships (Hillis, 1998), and that nuclear gene sequences have been offered as providing equally, if not more, realistic patterns of relationships, further investigation of the molecular systematics of the subfamilies of the Cyprinidae is in order. For these reasons, our analyses of relationships of this important and diverse family necessarily included extensive sampling of Asian cyprinid species and genera to hopefully improve our understanding and the resolution of cyprinid relationships.

In this study, we examined DNA sequence data from the nuclear recombination activating gene 2 (RAG2) to infer phylogenetic relationships within the East Asian cyprinids. The RAG2 gene encodes components of the recombinase involved in recombination of immunoglobin and T-cell receptor genes and appears as conserved single copies in all examined vertebrates (Hansen and Kaattari, 1996; Willett et al., 1997). The RAG2 gene has been widely used to evaluate intrageneric and intraspecific relationships (Baker et al., 2000; Clements et al., 2004; Hardman, 2004; Lewis-Oritt et al., 2001; Lovejoy and Collette, 2001), and it is also used to reveal higher-level phylogenetic relationships (Brinkmann et al., 2004; Calcagnotto et al., 2005). Although the considerable morphological variability of East Asian cyprinids represents a challenge to phylogenetic analyses based on morphology, we included representative species from all hypothesized subfamilies (Chen, 1998) in our present molecular analysis.

The aims of this study were (1) to identify monophyletic groups within the East Asian Cyprinidae, (2) to assess support for the currently recognized subfamilies using RAG2 sequence data, (3) to investigate whether the RAG2 gene could provide greater resolution of the high level relationships within Cyprinidae, (4) and to date the time of origin of the recovered clades.

2. Materials and methods

2.1. Sample collection

For this study, novel RAG2 gene sequences were determined for 113 species of fishes, including four outgroup taxa (non-cyprinid cypriniform outgroups), and 109 cyprinid species selected from all taxonomic subfamilies represented by Chen (1998) (Table 1). In addition to the 113 newly determined sequences, the RAG2 sequences of *Danio rerio* (NM131385), *Gyrinocheilus* sp. (AY804074), *Misgurnus* sp. (AY804103), and *Puntius tetrazona* (AY804121) were downloaded from GenBank. Most specimens used in this study were collected from a variety of locations in China (Table 1) and are deposited in the Fish Collection of the Institute of Hydrobiology of the Chinese Academy of Sciences. Muscle or fin tissue was preserved in 95% ethanol. The assigned outgroup taxa included the following six species from four families, also from the order Cypriniformes: *Misgurnus* sp., *Micronemacheilus pulcher*, and *Paramisgurnus dabryanus* (Cobitidae), *Myxocyprinus asiaticus* (Catostomidae), *Gyrinocheilus* sp. (Gyrinocheilidae), and *Pseudogastromyzon fangi* from Balitoridae/Homalopteridae.

2.2. DNA extraction, PCR amplification and sequencing

Total DNA was extracted from muscle or fin tissues using phenol/chloroform extraction procedure (Sambrook et al., 1989). RAG2 was amplified from total DNA extracts using the polymerase chain reaction (PCR). Primers RAG2-f2a and RAG2-R6a were adapted from Lovejoy and Collette (2001). Reaction mixtures contained approximately 100 ng of DNA template, 5 μL of 10× reaction buffer, 2 μL dNTPs (each 2.5 mM), 2.0 U *Taq* polymerase, and 1 μL of each oligonucleotide primer, each at 10 μM concentration, in a final volume 50 μL. The PCR amplification profile included an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation of 30 s at 94 °C, annealing of 30 s at 55 °C, extension of 90 s at 72 °C, and a final extension of 8 min at 72 °C. Amplified DNA was fractionated by

Table 1
Samples of cyprinid ingroup and outgroup taxa collected in this study

Subfamily	Taxa	Sampling location	Accession No.
Barbinae	*Acrossocheilus beijiangensis*	Rong'an, Guangxi Prov.	DQ366967
	Acrossocheilus elongatus	Rong'an, Guangxi Prov.	DQ366979
	Acrossocheilus hemispinus	Rong'an, Guangxi Prov.	DQ366986
	Balantiocheilos melanopterus	Aquarium, Wuhan	DQ366933
	Barbodes huangchuchieni	Mengla, Yunnan Prov.	DQ366952
	Barbodes vernayi	Mengla, Yunnan Prov.	DQ366987
	Barbodes wynaadensis	Houqiao, Yunnan Prov.	DQ366944
	Barbonymus schwanenfeldii	Aquarium, Wuhan	DQ366961
	Barbus barbus	France	DQ366990
	Barbus sp.	Africa	DQ366980
	Hampala macrolepidota	Mengla, Yunnan Prov.	DQ366965
	Onychostoma barbatula	Fuan, Fujian Prov.	DQ366964
	Onychostoma gerlachi	Jinghong, Yunnan Prov.	DQ366963
	Onychostoma leptura	Xilin, Guangxi Prov.	DQ366955
	Onychostoma macrolepis	Taian, Shandong Prov.	DQ366942
	Onychostoma ovale rhomboides	Tain'e, Guangxi Prov.	DQ366988
	Onychostoma rara	Tain'e, Guangxi Prov.	DQ366984
	Onychostoma sima	Hejiang, Sichuan Prov.	DQ366991
	Percocypris pingi pingi	Hejiang, Sichuan Prov.	DQ366962
	Puntius semifasciolatus	Jinghong, Yunnan Prov.	DQ366951
	Puntius tetrazona varieties	Aquarium, Wuhan	DQ366938
	Sikukia stejnegeri	Mengla, Yunnan Prov.	DQ366931
	Sinocyclocheilus tingi	Fuxian Lake, Yunnan Prov.	DQ366978
	Spinibarbus hollandi	Tunxi, Anhui Prov.	DQ366973
	Tor douronensis	Menglun, Yunnan Prov.	DQ366945
	Tor qiaojiensis	Yingjiang, Yunnan Prov.	DQ366970
	Tor sinensis	Mengla, Yunnan Prov.	DQ366936
Cyprininae	*Carassius auratus*	Wuhan, Hubei Prov.	DQ366941
	Cyprinus carpio	Tain'e, Guangxi Prov.	DQ366994
	Cyprinus multitaeniata	Guiping, Guangxi Prov.	DQ366939
	Procypris rabaudi	Hejiang, Sichuan Prov.	DQ366969
Labeoninae	*Cirrhinus molitorella*	Tengxian, Guangxi Prov.	DQ366959
	Crossocheilus latius	Tengchong, Yunan Prov.	DQ366982
	Crossocheilus reticulatus	Menglun, Yunnan Prov.	DQ366937
	Discogobio bismargaritus	Liuzhou, Guangxi Prov.	DQ366947
	Discogobio brachyphysallidos	Jinxiu, Guangxi Prov.	DQ366958
	Discogobio laticeps	Tain'e, Guangxi Prov.	DQ366949
	Epalzeorhynchos frenatus rar	Aquarium, Jinghong	DQ366943
	Garra kempi	Chayu, Xizang Prov.	DQ366968
	Garra mirofrontis	Tengchong, Yunnan Prov.	DQ366934
	Garra orientalis	Ledong, Hainan Prov.	DQ366957
	Garra pingi	Mengla, Yunnan Prov.	DQ366972
	Garra taeniata	Jinghong, Yunnan Prov.	DQ366953
	Henicorhynchus lineatus	Menglun, Yunnan Prov.	DQ366935
	Labeo yunnanensis	Mengla, Yunnan Prov.	DQ366948
	Lobocheilus melanotaenia	Menglun, Yunnan Prov.	DQ366940
	Osteochilus salsburyi	Rong'an, Guangxi Prov.	DQ366971
	Parasinilabeo assimilis	Rong'an, Guangxi Prov.	DQ366992
	Pseudocrossocheilus bamaensis	Tain'e, Guangxi Prov.	DQ366993
	Ptychidio jordani	Tain'e, Guangxi Prov.	DQ366974
	Rectoris luxiensis	Luxi, Hunan Prov.	DQ366977
	Rectoris posehensis	Dou'an, Guangxi Prov.	DQ366975
	Semilabeo notabilis	Jinxiu, Guangxi Prov.	DQ366983
	Sinilabeo rendahli	Yidu, Hubei Prov.	DQ366932
Schizothoracinae	*Gymnocypris e. eckloni*	Huanghe, Qinghai Prov.	DQ366950
	Gymnocypris p. przewalskii	Qinghai Lake, Qinghai Prov.	DQ366954
	Gymnodiptychus dybowskii	Yili, Xinjiang Prov.	DQ366956
	Schizopygopsis y. younghusbandi	Bomi, Xizang Prov.	DQ366976
	Schizothorax dulongensis	Guyong, Yunnan Prov.	DQ366985
	Schizothorax meridionalis	Yingjiang, Yunnan Prov.	DQ366989
	Schizothorax molesworthi	Chayu, Xizang Prov.	DQ366946

(*continued on next page*)

Table 1 (*continued*)

Subfamily	Taxa	Sampling location	Accession No.
	Schizothorax myzostomus	Guyong, Yunnan Prov.	DQ366960
	Schizothorax waltoni	Chayu, Xizang Prov.	DQ366981
Leuciscinae	*Cyprinella lutrensis*	GN531	DQ367019
	Leuciscus leuciscus	France	DQ367007
	Phoxinus phoxinus	Europe	DQ367022
	Phoxinus lagowskii	Hengren, Liaoning Prov.	DQ367035
	Rutilus rutilus	France	DQ367003
	Pimephales promelas	GN530	DQ367000
	Rhinichthys atratulus	GN529	DQ367018
	Elopichthys bambusa	Taoyuan, Hunan Prov.	DQ367016
	Ochetobius elongatus	Taoyuan, Hunan Prov.	DQ367012
	Luciobrama macrocephalus	Tengxian, Guangxi Prov.	DQ367013
	Ctenopharyngodon idella	Hengxian, Guangxi Prov.	DQ366996
	Mylopharyngodon piceus	Taoyuan, Hunan Prov.	DQ367011
	Squaliobarbus curriculus	Wuhan, Hubei Prov	DQ367021
	Tinca tinca	Europe	DQ367029
Hypophthalmichthyinae	*Hypophthalmichthys molitrix*	Chenxi, Hunan Prov.	DQ367002
	Aristichthys nobilis	Wuhan, Hubei Prov.	DQ367038
Xenocyprinae	*Distoechodon tumirostris*	Wuhan, Hubei Prov.	DQ366998
	Pseudobrama simoni	Taoyuan, Hunan Prov.	DQ367028
	Xenocypris argentea	Taoyuan, Hunan Prov.	DQ367024
Danioninae	*Danio apogon*	Mengla, Yunnan Prov.	DQ367039
	Hemigrammocypris rasborella	Japan	DQ367008
	Nicholsicypris normalis	Diaoluoshan, Hainan Prov.	DQ367034
	Opsariichthys bidens	Taoyuan, Hunan Prov.	DQ367014
	Raiamas guttatus	Mengla, Yunnan Prov.	DQ366966
	Tanichthys albonubes	Aquarium, Wuhan	DQ367023
	Zacco platypus	Jinxiu, Guangxi Prov.	DQ367010
Cultrinae	*Culter alburnus*	Taoyuan, Hunan Prov.	DQ367004
	Megalobrama amblycephala	Wuhan, Hubei Prov.	DQ367025
	Sinibrama macrops	Rong'an, Guangxi Prov.	DQ367006
	Pseudohemiculter dispar	Rong'an, Guangxi Prov.	DQ367001
	Pseudolaubuca sinensis	Taoyuan, Hunan Prov.	DQ367017
	Rasborinus lineatus	Hengxian, Guangxi Prov.	DQ367036
	Cultrichthys erythropterus	Lingshan, Guangxi Prov.	DQ367037
	Toxabramis swinhonis	Bobai, Guangxi Prov.	DQ367027
Gobiobotinae	*Gobiobotia abbreviata*	Tain'e, Guangxi Prov.	DQ367033
	Gobiobotia filifer	Wuhan, Hubei Prov.	DQ367032
Gobioninae	*Abbottina rivularis*	Nanchong, Sichuan Prov.	DQ366995
	Coreius heterodon	Wuhan, Hubei Prov.	DQ367005
	Gobio gobio	France	DQ367015
	Pseudorasbora sp.	Aquarium, Jinghong	DQ367030
	Pseudogobio vaillanti	Tain'e, Guangxi Prov.	DQ366999
	Pseudorasbora parva	Jinxiu, Guangxi Prov.	DQ366997
	Sarcocheilichthys s. sinensis	Hejiang, Sichuan Prov.	DQ367026
	Saurogobio dabryi	Changyang, Hubei Prov.	DQ367020
Acheilognathinae	*Paracheilognathus meridianus*	Hengxian, Guangxi Prov.	DQ367009
	Rhodeus sp.	Xilin, Guangxi Prov.	DQ367031
Outgroup	*Micronemacheilus pulcher*	Rong'an, Gaungxi Prov.	DQ367041
	Myxocyprinus asiaticus	Wuhan, Hubei Prov.	DQ367043
	Paramisgurnus dabryanus	Rong'an, Guangxi Prov.	DQ367040
	Pseudogastromyzon fangi	Hengxian, Guangxi Prov.	DQ367042

The cyprinid subfamilies followed those proposed by Chen (1998).

electrophoresis through 0.8% low-melting agarose gels, recovered from the gels, and purified using BioStar Glassmilk DNA purification Kit according to manufacturer's instructions. Nucleotide sequences of RAG2 were determined using purified PCR product.

2.3. Sequence alignment

Multiple alignments of sequences were performed using CLUSTAL X (Thompson et al., 1997). All sequences have been deposited in GenBank (Table 1).

Measures of nucleotide composition were obtained using the program PAUP* 4.0b10 (Swofford, 2003). Base composition was calculated across all taxa, for 1st, 2nd, and 3rd codon positions and all codon positions combined. A chi-square (χ^2) test of base heterogeneity was calculated for each codon position and for all codon positions, as implemented in PAUP* 4.0b10. As a heuristic tool to explore the degree of saturation present in the datasets, we plotted raw sequence divergence (uncorrected *p* distance) vs. number of transition and transversion substitutions for all pairwise comparisons among taxa, for all codon positions as a whole and 3rd positions only (Fig. 1). If the codon position sites were saturated, we would expect to see a plateau in such a plot, where little or no additional substitution is detectable with increased *p* distance. Because no such plateaus are seen (Fig. 1), we conclude that saturation has not yet occurred in 1st, 2nd, and 3rd position sites. Therefore, we did not exclude characters or employ a weighting scheme in our parsimony analyses.

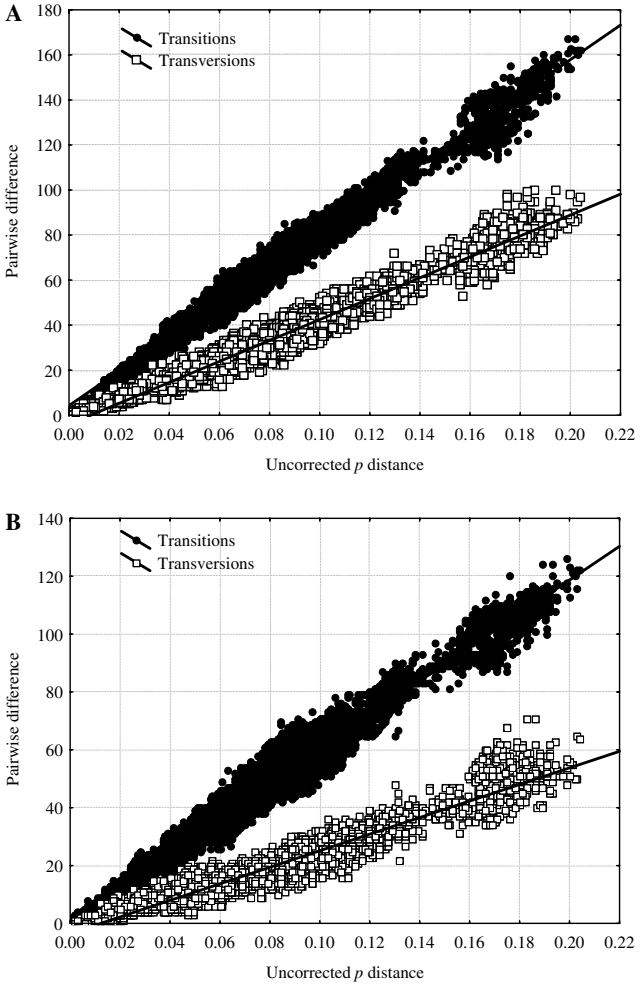

Fig. 1. Number of transition and transversion substitutions vs. the uncorrected *p* distance for the RAG2 genes for (A) all position and (B) 3rd position sites.

2.4. Phylogenetic analysis: parsimony and likelihood

Phylogenetic analysis was conducted with maximum parsimony (MP) method using PAUP* 4.0b10. Heuristic searches were performed using tree bisection–reconnection (TBR) branch-swapping and 10 random sequence addition replicates. All sites were equally weighted and gaps were treated as missing characters. Support for recovered clades was measured using a nonparametric bootstrap analysis (Felsenstein, 1985) with 1000 total pseudoreplicates and a heuristic search with TBR branch swapping and two random sequence addition replicates per pseudoreplicate.

For maximum likelihood (ML) analysis, Modeltest 3.7 (Posada and Crandall, 1998) was used to determine the optimal model of nucleotide evolution. The $\mathrm{TrN}+\mathrm{I}+\Gamma$ substitution model (Base = 0.2907, 0.2324, 0.2059; Rmat = 1.0000, 4.3874, 1.0000, 1.0000, 4.9986) with invariable sites (pinvar = 0.3624) and among-site rate heterogeneity ($\alpha = 1.1226$) was selected using a set of hierarchical likelihood ratio tests (LRTs) implemented in Modeltest. The ML method was then performed to find the optimal ML tree with a heuristic search as implemented in PAUP* 4.0b10, with TBR branch-swapping and 10 random sequence additions. Support for recovered clades was assessed using the bootstrap analysis with 100 total pseudoreplicates by using the program PHYML (Guindon and Gascuel, 2003).

2.5. Bayesian analysis

Bayesian analyses (BA) were carried out with MrBayes 3.0 (Ronquist and Huelsenbeck, 2003) to calculate posterior probabilities of recovered clades, with the optimal model of sequence evolution determined from the LRTs. MrBayes 3.0 was run with 1×10^6 generation Markov chain. Starting trees were random, and one cold and three heated chains were run simultaneously. Trees were saved every 100 generations for a total size of 10,000 in the initial sample. Graphical inspection of tree log-likelihood in this sample revealed that the stationarity was reached within 100,000 generations. Thus, we discarded the first 100,000 generations (1000 sampled trees) as burn-in and used the remaining 900,000 generations (9000 sampled trees) in all subsequent analysis. A majority rule consensus tree calculated from the 9000 remaining trees was used to determine the posterior probabilities of clades.

2.6. Molecular clock test and divergence time estimation

Likelihood ratio test was performed to investigate whether a global molecular clock fitted the RAG2 dataset and PAUP* 4.0b10 was used to obtain the likelihood scores of each phylogeny. A significant difference was observed between the likelihood scores of clock and non-clock model ($\chi^2 = 376.686$, df = 115, $P < 0.05$). Therefore, nonparametric rate smoothing (NPRS) method (Sanderson, 1997) as implemented in r8s (Sanderson, 2003) was applied to

estimate divergence times. Powell's method for optimizing the objective function and the log penalty function were used.

Divergence times were estimated using the tree topology resulting from the ML analysis. To calculate divergence times for the nodes in the tree, it is necessary to calibrate the divergence time of at least one node in the tree. In the present study, we adopted two ways to calibrate the divergence time. One method is that the recent literature-based separation of the subfamilies of Cyprininae and Leuciscinae occurred in the mid-Oligocene (27.70 MYA) (Zardoya and Doadrio, 1999) and this was fixed to estimate the divergence time of these nodes. The second method included fossil-based calibration points. The oldest and reliable fossils of Cyprinidae are known from mid-Eocene (Cavender, 1991) and thus the root node of the Cyprinidae was dated to Eocene and was constrained by a maximum age of 55.8 MYA. The minimum age constraints (1.81 MYA) are according to the fossil records of extent cyprinids in Pliocene in China (Liu and Su, 1962). A minimum age of 3 MYA was assigned to the node leading to the genus *Pseudorasbora* based on the fossil record of *Pseudorasbora* (Liu and Su, 1962).

3. Results

3.1. The RAG2 gene sequences and variations

The RAG2 gene sequences obtained from the 113 sampled fish species ranged in size from 1170 to 1284 bp, and the aligned sequences consisted of 1287 nucleotide sites. An autapomorphic insertion of three base pairs (one codon) in *Phoxinus phoxinus* and an autapomorphic deletion of six base pairs (two codons) in *Osteochilus salsburyi* were found within the RAG2 gene. Of the 1287 bp nucleotide sites, 600 characters were identical among all taxa, 684 sites were variable, and 563 were parsimony informative. Average percentage sequence divergence (uncorrected *p* distance) within cyprinid species was 7.8%, and maximum ingroup RAG2 divergence was 15.3% (between *Danio apogon* and *Garra orientalis*, and between *Danio rerio* and *Osteochilus salsburyi*). The highest sequence divergences were between ingroup cyprinids and the outgroup taxon *Micronemacheilus pulcher*, with maximum sequence divergence ranging as high as 20.3%. Average sequence divergence among outgroup taxa analyzed was 14.5%. The sequence divergence within the East Asia endemic cyprinid species, including silver carp (*Hypophthalmichthys*), big-head carp (*Aristichthys*), grass carp (*Ctenopharyngodon*), black carp (*Mylopharyngodon*), *Ochetobius*, barbel chub (*Squaliobarbus*), yellowcheek (*Elopichthys*), *Luciobrama*, as well as the cultrins [i.e. *Culter*, *Cultrichthys*, *Pseudohemiculter*, *Pseudolaubuca*, *Sinibrama*, *Toxabramis*, and Wuchang bream (*Megalobrama*)], and the xenocyprins [i.e., *Distoechodon*, *Pseudobrama*, and yellowfin (*Xenocypris*)], was 0.6–3.7%.

Mean base composition was found to be fairly uniform among all taxa analyzed (23.73% A, 27.10% C, 25.39% G, and 23.78% T), with a slightly higher proportion of guanine (32.43%) and lower proportion of thymine (19.77%) at 1st codon positions. Third codon positions revealed relative reductions in the frequency of adenine and guanine (17.89%, 19.38%, respectively), but had slightly elevated amounts of cytosine and thymine (34.63%, 28.09%, respectively). Nucleotide composition among all taxa exhibited no significant heterogeneity at all three codon positions: first position, $\chi^2 = 22.91$ (df = 348, $P = 1.00$); second position, $\chi^2 = 15.25$ (df = 348, $P = 1.00$); and third position, $\chi^2 = 116.29$ (df = 348, $P = 1.00$).

The overall transition to transversion (Ti/Tv) ratio was 1.95, with 480,967 transitions and 246,250 transversions in the RAG2 dataset. The relationships between uncorrected *p* distance and number of transition and transversion substitutions of the RAG2 sequences were plotted for all pairwise species comparisons (including outgroup taxa), for all positions and for 3rd positions. All plots indicated that, even at 3rd codon positions, no saturation was found in the RAG2 genes (Fig. 1).

3.2. Phylogenetic relationships

Unweighted parsimony analysis resulted in 1800 equally parsimonious trees [tree length = 2856, consistency index (CI) = 0.3825, retention index (RI) = 0.7533]; the 50% majority-rule consensus tree is shown in Fig. 2. In the MP tree, the Cyprinidae is monophyletic, with 100% bootstrap support. Within the Cyprinidae, *Danio* emerged at first (Clade III, Fig. 2), and the remaining cyprinid species clustered into two major clades (Clade I and Clade II, Fig. 2) supported with strong bootstrap scores (98% and 92%, respectively). Within clade I, monophyly of Labeonini was strongly corroborated with 100% bootstrap support, whereas monophyly of Cyprinini (including barbins and schizothoracins) was not supported because of the low bootstrap value for the corresponding node (43%). The bootstrap analysis shows generally high support value for all recovered lineages within Clade II, with three exceptions: (1) the node supporting the sister group relationship of Gobionini + Acheilognathini and Leuciscini + Tincini (58%); (2) the node supporting the sister group relationship of Acheilognathini and Gobionini (37%); and (3) the node supporting the sister group relationship of Leuciscini and Tincini (35%). The endemic cyprinid taxa in East Asia, (including *Hypophthalmichthys*, *Aristichthys*, *Ctenopharyngodon*, *Mylopharyngodon*, *Ochetobius*, *Squaliobarbus*, *Elopichthys*, *Luciobrama*, *Culter*, *Cultrichthys*, *Sinibrama*, *Megalobrama*, *Pseudohemiculter*, *Toxabramis*, *Pseudolaubuca*, *Distoechodon*, *Xenocypris*, and *Pseudobrama*) formed a large monophyletic group with strong support (90%), and the genera *Nicholsicypris*, *Rasborinus*, *Hemigrammocypris*, *Zacco* and *Opsariichthys* were resolved as the basal members of this clade.

The ML tree (Fig. 3) was identical to the MP tree, with the following exceptions: (1) the phylogenetic position of

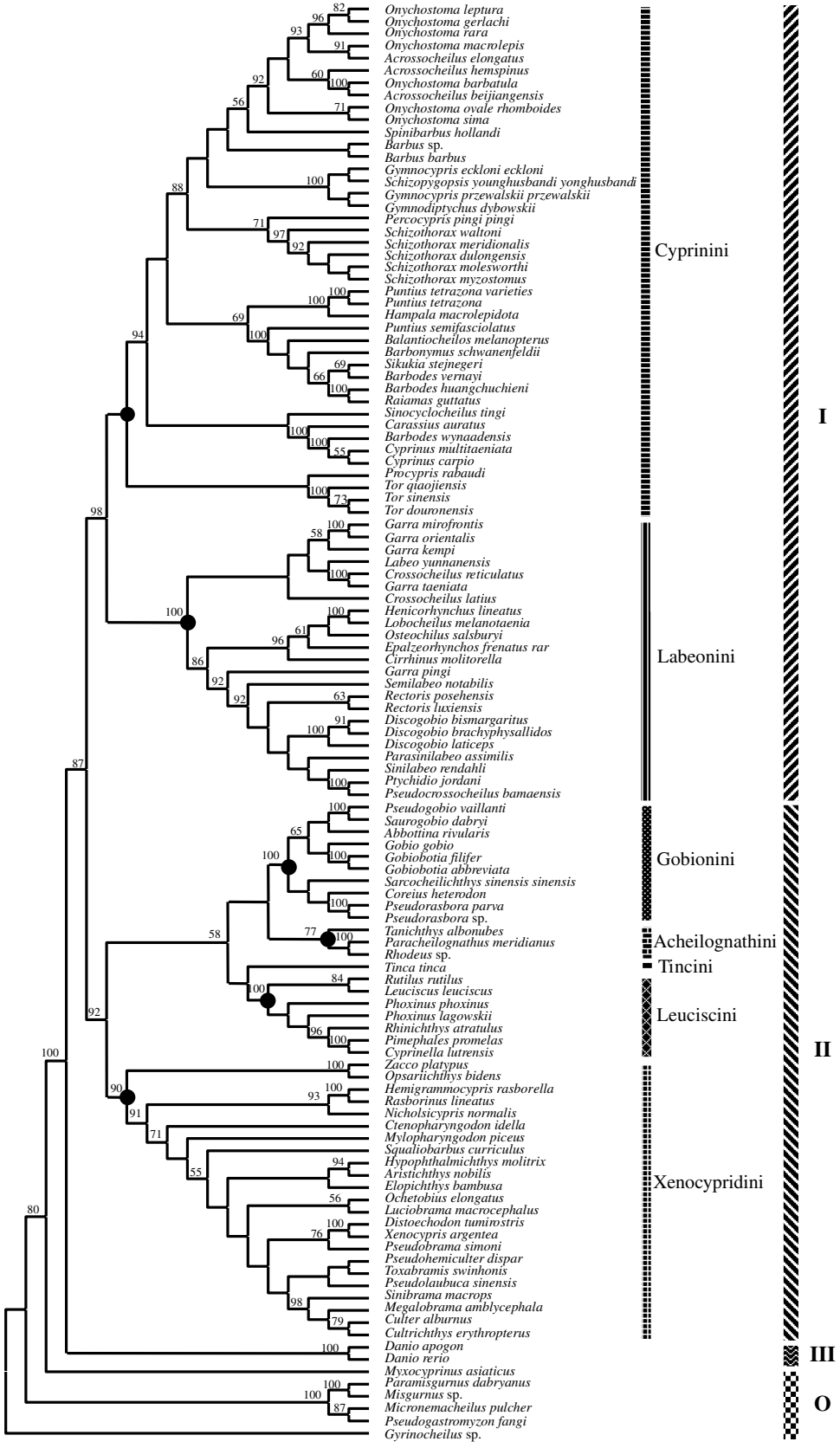

Fig. 2. The 50% majority-rule consensus of 1800 trees resulting from maximum parsimony analysis of the RAG2 gene dataset. Numbers at nodes represent percent recovery in bootstrap analysis (1000 replicates). Tree length = 2856; consistency index (CI) = 0.3825; retention index (RI) = 0.7533. Recognized clades are indicated by Roman numerals on the right side of the figure. (I) Cyprininae; (II) Leuciscinae; (III) Danioninae; (O) outgroup. Nodes for the recognized subclades are marked with black dot.

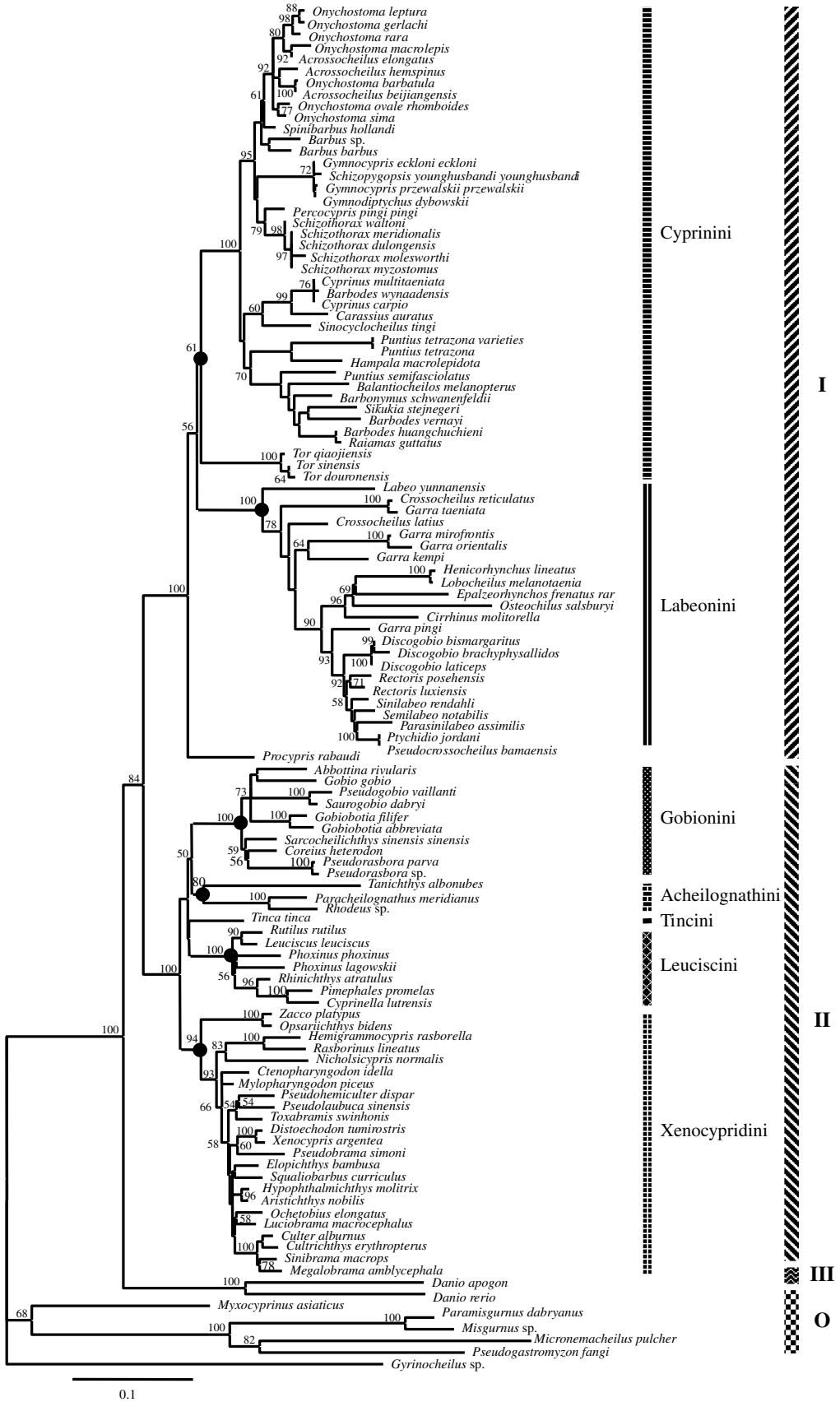

Fig. 3. Tree resulting from maximum likelihood analysis of the RAG2 gene dataset. −Ln likelihood = 16851.84111. Numbers at nodes represent percent recovery in bootstrap analysis (100 replicates). Recognized clades are indicated by Roman numerals on the right side of the figure (representations of Roman numerals follow that in Fig. 2), and nodes for the recognized subclades are marked with black dot.

Procypris rabaudi and (2) the deep branch pattern within the recognized tribes.

The BA tree (Fig. 4) is identical in topology to the ML tree. In the BA tree, the nodes are well resolved and most nodes for the recovered tribes were highly supported with significant posterior probabilities. Support for the sister group relationship of the tribes Acheilognathini and Gobionini was relatively low (75%) as was also the support for the grouping of the tribes Gobionini, Acheilognathini, Leuciscini, and Tincini (84%).

3.3. Dating main phylogenetic events

The ML topology was used for dating phylogenetic events because of the relatively well-resolved phylogenetic relationships. We assumed that the calibration point corresponding to the emergence of Cyprinidae is located at the node CYD (Fig. 5). The chronogram obtained for the family Cyprinidae is provided in Fig. 5. Table 2 provides divergence time estimates of the main divergences within the family. Using the fossil-based calibration points, the root node of the cyprinid revealed a divergence of time of 46.82 MYA. The first cladogenesis within the Cyprinidae dates to 30.12 MYA. An age estimate of 26.08 MYA was assign to the node leading to separation of the two primary lineages, Cyprininae and Leuciscinae. While we used two different calibration sources (literature-based estimates and fossils), no obvious differences were found between these two results (Table 2).

4. Discussion

4.1. Monophyletic clades within the East Asian cyprinids

The current analysis includes the most extensive published taxonomic sampling of proposed major lineages in the family to date, as well as the use of the largest nuclear DNA dataset of a new nuclear marker, the RAG2 gene, in elucidating the phylogenetic relationships in Cyprinidae and the proposed subfamilies. From this analysis, the most significant result regarding the phylogenetic relationships of cyprinids is the demonstration of monophyly of the family, the monophyly of both the subfamilies Cyprininae and Leuciscinae, and the tribes Labeonini, Gobionini, Acheilognathini, Leuciscini, and Xenocypridini.

Our resolved monophyly of the Cyprininae (sensu Howes, 1991) is well supported and in agreement with the previous morphological (Cavender and Coburn, 1992; Chen et al., 1984) and molecular studies (Gilles et al., 1998, 2001; He et al., 2004; Liu and Chen, 2003). Based on previous morphological data, the Cyprininae could be subdivided into four subfamilies, e.g., Barbinae, Cyprininae, Labeoninae, and Schizothoracinae (Chen, 1998). On the whole, results from sequence variation in the RAG2 data provide robust evidence only for monophyly of the currently recognized Labeoninae; the monophyly of the Cyprininae, Barbinae, and Schizothoracinae remain unsupported. Based on our present study, only two monophyletic clades can be identified within the Cyprininae, the strongly supported Labeonini and the weakly supported Cyprinini (including barbins and schizothoracins, and perhaps exclusive of *Procypris*).

The subfamily Leuciscinae is another primary clade within the family Cyprinidae. Phylogenetic analyses of the RAG2 sequence variation provides substantial resolution and support for the monophyly of the Leuciscinae and four tribes, such as Gobionini (including *Gobiobotia*), Acheilognathini, Leuciscini, and Xenocyprinidini,. All the leuciscine tribes, except Xenocyprinidini, were once referred to as subfamilies of the Cyprinidae by morphological (Chen, 1998; Chen et al., 1984) and recent molecular studies (Gilles et al., 1998, 2001; Liu and Chen, 2003).

The most striking result in current study is the fact that the endemic taxa in East Asia are closely related to each other and form a monophyletic group, the Xenocyprinidini. This tribe was named Xenocyprinidini because it was first used for this group by Günther (1868) and was subsequently recommended as a formal group by Liu and Chen (2003). Given the considerable morphological diversity in this group, taxa within the tribe Xenocyprinidini were usually assigned to separate cyprinid subfamilies by traditional taxonomy (Chen, 1998; Chen et al., 1984; Howes, 1991). Although bootstrap support is low, the close relationships among species within the Xenocyprinidini were suggested by the recent molecular studies (He et al., 2004; Liu and Chen, 2003). In contrast, the RAG2 gene is particularly useful for monophyly of the tribe Xenocyprinidini (with MP and ML bootstrap value and posterior probability support of 90%, 94%, and 100%, respectively). A reasonable interpretation of the performance of the RAG2 gene in providing this strong support is its lower rate of evolution and its function in immunological response is not linked to morphological features that have been traditionally used as the diagnostic criteria for higher-level classifications in the Cyprinidae. Therefore, we hypothesize that the East Asian endemic cyprinids, including silver carp, bighead carp, grass carp, black carp, *Ochetobius*, barbel chub, yellowcheek, *Luciobrama*, as well as the cultrins and the xenocyprins, share the more recent common ancestor with the small group including *Nicholsicypris*, *Rasborinus*, *Hemigrammocypris*, *Zacco* and *Opsariichthy*.

Morphologically, Danioninae (Rasborinae sensu Howes, 1991) is a large assemblage containing mostly taxa not accommodated by the other subfamilies. The monophyly for Danioninae has been rejected by recent molecular studies based on mtDNA gene (Gilles et al., 1998; He et al., 2004; Liu and Chen, 2003), and the polyphyletic nature of the danionine group is also confirmed in present study. Due to difficulties of sampling appropriate taxa, we have included limited danionine taxa in our analysis. Based on the present and recent (He et al., 2004; Liu and Chen, 2003) molecular studies, redefinition of the

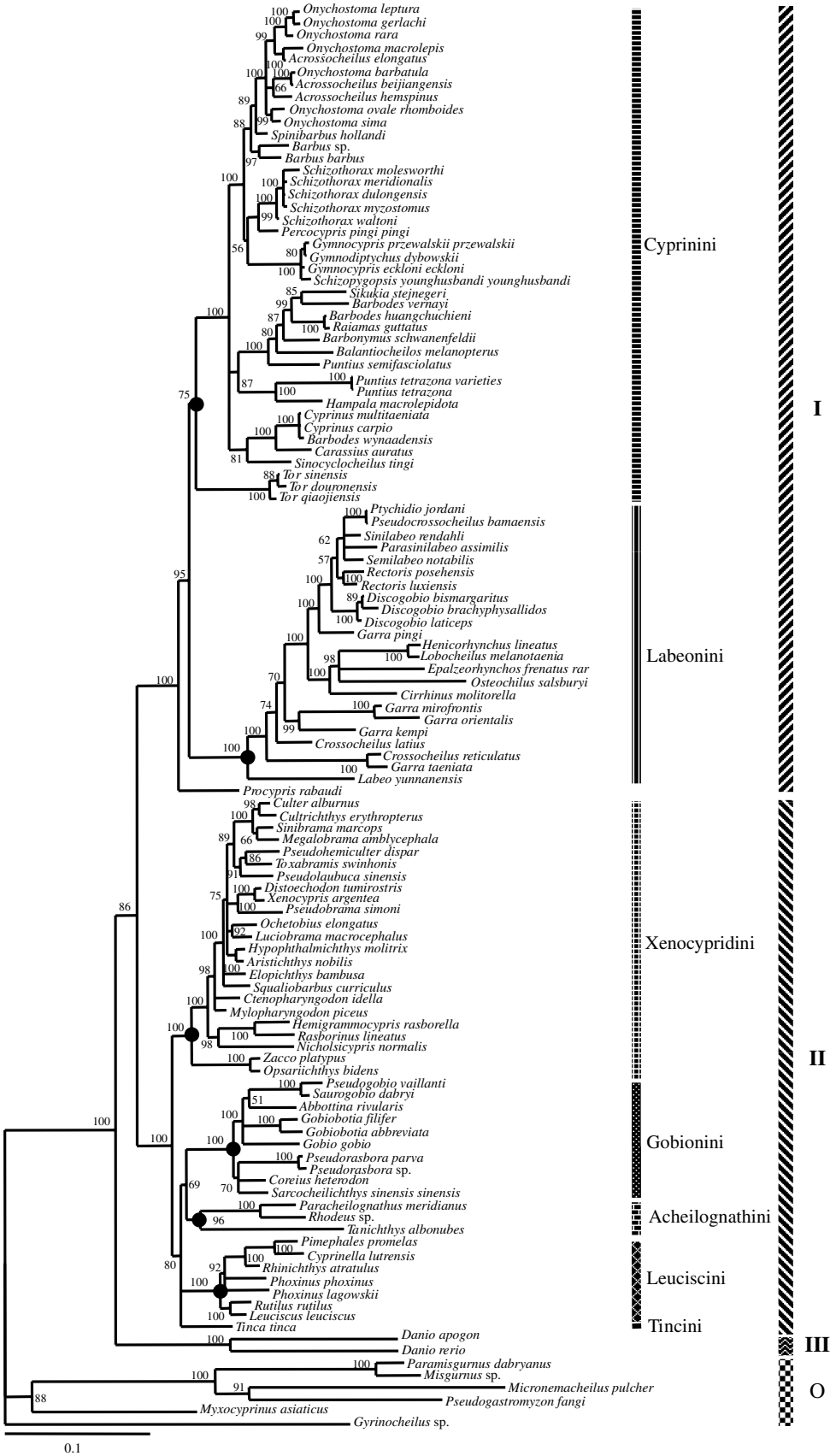

Fig. 4. The 50% majority-rule consensus tree resulting from Bayesian analysis of the RAG2 gene dataset. Numbers at nodes represent Bayesian posterior probabilities. Recognized clades are indicated by Roman numerals on the right side of the figure (representations of Roman numerals follow that in Fig. 2), and nodes for the recognized subclades are marked with black dot.

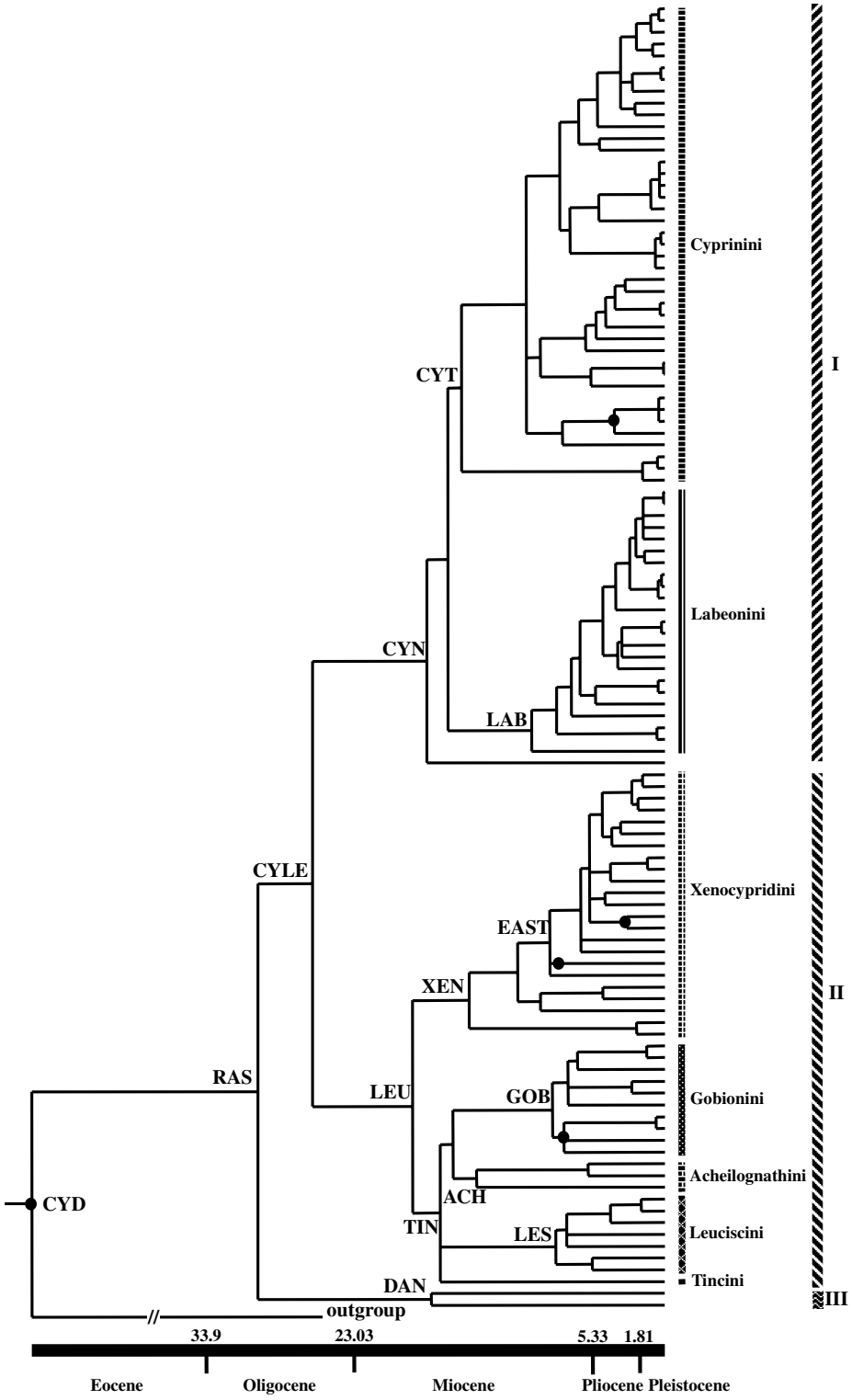

Fig. 5. Chronogram obtained from the RAG2 gene dataset for the family Cyprinidae, with ages estimated using nodes under the fossil-based constraints (indicated with black dots). Node symbols refer to those in Table 2. Representations of Roman numerals follow that in Fig. 2.

subfamily Danioninae could exclude the East Asia endemic taxa *Zacco*, *Opsariichthys*, and *Nicholsicypris*. Although herein we treat the redefined Danioninae as a subfamily, there is still a need for considerably more study and taxonomic revision of this complex and speciose group.

4.2. Phylogenetic relationships among cyprinids

It is striking to notice that historical morphological studies have usually supported an early split between two cyprinid lineages, e.g., barbelled cyprinine and usually non-barbel leuciscine (Chen et al., 1984; Howes, 1991). The trees

Table 2
Divergence time estimates of the main splits in the family Cyprinidae of Fig. 5

Node	Clade	Age estimates (MYA)	
		A	B
CYD	Cyprinidae and outgroup	49.76	46.82 (**55.80**)
RAS	Cyprinidae, except outgroup	31.99	30.12
CYLE	Cyprininae and Leuciscinae	**27.70**	26.08
CYN	Cyprininae	18.71	17.64
CYT	Cyprinini	15.96	15.06
LAB	Labeonini	10.49	9.94
LEU	Leuciscinae	19.83	18.65
TIN	Acheilognathini, Gobionini, Leuciscini, and Tincini	17.68	16.64
GOB	Gobionini	8.87	8.36
ACH	Acheilognathini	14.83	13.95
LES	Leuciscini	8.58	8.07
XEN	Xenocypridini	15.41	14.49
EAST	Xenocypridini, except *Hemigrammocypris*, *Nicholsicypris*, *Opsariichthys*, *Rasborinus*, and *Zacco*	9.07	8.52
DAN	*Danio*	18.38	17.31

A, ages estimated using literature-based calibration from Zardoya and Doadrio (1999) and Node CYLE fixed at 27.70 million years ago (MYA). B, ages estimated using fossil-based calibrations and a maximum age constraint of 55.80 MYA assigned to node CYD (in bracket). Ages used as calibrations are in bold.

resulting from phylogenetic analyses of the RAG2 gene place the Danioninae as the basal-most group in the family and divide the remaining cyprinids into two divergent subfamilies. The basal relationship of the Danioninae, relative to other Cyprinidae, has also been confirmed in other recent molecular analyses (Gilles et al., 1998, 2001).

In the classification based on morphology the position of *Tinca* was controversial (Cavender and Coburn, 1992; Chen et al., 1984) or was placed *incertae sedis* (Howes, 1991). Resolution of the phylogenetic position of *Tinca* within the Cyprinidae has also proved difficult in recent molecular studies (Briolay et al., 1998; Gilles et al., 1998; Liu and Chen, 2003; Zardoya and Doadrio, 1999). Unlike previous analyses, however, the trees resulting from the RAG2 sequences support *Tinca* as a tribe Tincini with high nodal support. Although the close grouping of the tribes Tincini, Leuciscini, Gobionini, and Acheilognathini is not supported with high bootstrap values or posterior probabilities, it is likely that Tincini is in a clade inclusive of Leuciscini plus Gobionini plus Acheilognathini.

While the deep-branch relationships among the taxa within the leuciscine tribe Xenocypridini remain unsolved because of minimal sequence variation in these species, the RAG2 gene trees clearly place the genera *Zacco* and *Opsariichthys* as basal members of this tribe. We hypothesize that the minimal variation of the RAG2 gene sequences in this group of endemic East Asia cyprinid species of Xenocypridini (uncorrected *p* distance, 0.6–3.7%) is the reason for the low values of bootstrap support in the MP and ML analyses (Figs. 2 and 3) and somewhat low posterior probability in BA (Fig. 4).

In conclusion, based on sequence variation in the RAG2 gene the relationships among the major lineages of the Cyprinidae can be described in terms of the tree shown in Fig. 6. The subfamily Danioninae is resolved as the basal-most subfamily within the Cyprinidae. The monophyletic Cyprininae includes the monophyletic Labeonini and the

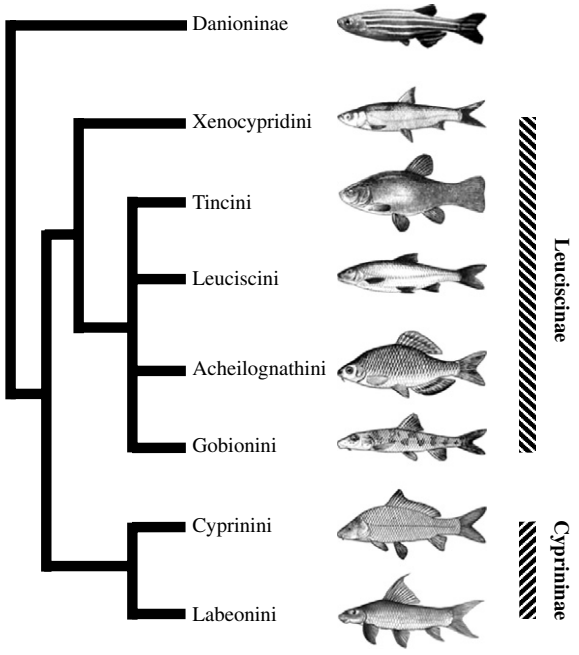

Fig. 6. Cyprinid intrarelationships inferred from phylogenetic analysis of the RAG2 gene dataset.

likely non-monophyletic Cyprinini. Within the monophyletic Leuciscinae, five monophyletic tribes can be recognized. Although the relationships among Tincini, Leuciscini, Gobionini, and Acheilognathini are not well supported, these four tribes are closely grouped.

4.3. Time of origin of cyprinid clades and evolutionary scenario

The family Cyprinidae likely originated at around 46–49 MYA (Fig. 5 and Table 2). This RAG2-estimated origin of cyprinids is older than the 39 MYA based on the cytochrome *b* (Zardoya and Doadrio, 1999). The later origin of

cyprinids based on the cytochrome *b* is likely an underestimation due to saturation in cytochrome *b*. Therefore, both the present molecular dating analyses and the fossil records of the family in Eurasia (Cavender, 1991) indicate that cyprinids may have originated in the mid-Eocene in Asia. The cladogenetic event leading to the divergence of the common ancestor to Danioninae may have occurred in the early Oligocene (about 30–31 MYA), and the origins of Cyprininae and Leuciscinae would have occurred in the mid-Oligocene (about 26 MYA).

Our molecular dating analyses support an early Oligocene origin for the Danioninae and an early Miocene speciation for the genus *Danio* (about 17–18 MYA). The origin and diversification of Danioninae are compatible with the morphological hypothesis that danionine fishes are primitive (Chen, 1998; Chen et al., 1984). To further corroborate the basal position of Danioninae within Cyprinidae, future analyses should include additional danionines from tropical Asia and Africa.

The Cyprininae includes at least two groups, Labeonini and Cyprinini. The recent species of this clade have a very wide distribution in southern China, Southeast Asia, South Asia (especially in India), southern Europe, and Africa. Based on zoogeographic evidence, Gosline (1978) suggested that modern cyprinids appeared to have evolved from a cyprinine-like ancestor. The current analyses do not support this hypothesis given the observed basal position of Danioninae and the observation that the origins of Leuciscinae and Cyprininae occurred almost simultaneously in the early Miocene (about 19 and 18 MYA, respectively) (Fig. 5 and Table 2). Within the Cyprininae, the Cyprinini experienced an early radiation in the mid-Miocene (about 15 MYA), while the Labeonini experienced a later radiation in the late Miocene (about 10 MYA).

The early Miocene radiation (about 19 MYA) of the Leuciscinae ultimately led to the division of this subfamily into two major groups, one consisting of the clades Leuciscini, Tincini, Gobionini, and Acheilognathini, and the other including only Xenocyprinidini. Within the former group the common ancestor to Tincini diverged in the early Miocene (around 17 MYA), while the Acheilognathini experienced a mid-Miocene radiation (about 14 MYA) and the Gobionini and Leuciscini clades arose later in the late Miocene (about 8 MYA). Members of Leuciscini, Tincini, Gobionini, and Acheilognathini, in part, have a northern distribution in North America and Eurasia. Because of their adaptation to higher-latitude freshwater habitats, Chen (1998) referred to these groups as northern cold-water lineage.

A large and diverse East Asian clade of Cyprinidae, Xenocyprinidini, is strongly supported by the RAG2 data. The radiation of this major East Asian cyprinid clade is estimated to have taken place in the mid-Miocene (about 15 MYA). The node EAST (Fig. 5) represents a group of endemic East Asia cyprinids that Chen (1998) referred to as the East Asian group that includes about 29 genera and 81 species. The distribution of all of these species is restricted to river drainages and lakes in East Asia, especially in China. According to our molecular dating, species divergences of the East Asian group should have occurred from the late Miocene to the Pleistocene (1.3–9 MYA, data not shown). During almost the same period, from the late Miocene to the Pliocene, the evolution of Asian monsoons underwent three stages of development and the intensification of the East Asian winter monsoon was thought to have occurred since about 2.6 MYA (An et al., 2001). One remarkable characteristic of this East Asian group of cyprinids is that the reproduction of most taxa within this group is affected by the East Asian monsoons. These East Asian cyprinids must enter into the rivers to spawn during the rainy season that accompanies the East Asian summer monsoon. Interestingly, the estimated ages of the divergences within this endemic East Asian cyprinid group overlaps significantly with, and may be linked to, the environmental changes in freshwater habitats where these species were distributed during this period of climatic change and the monsoon evolution. Further testing of this hypothesis using additional taxa from this clade and additional genes is expected to lead to a better understanding of the consequences climatic change on the diversification of this diverse and endemic clade of Cyprinidae.

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